

RESEARCH COMMUNICATION

TISSUE CULTURE STUDIES ON A SUSPECTED LYSOSOMAL STORAGE DISEASE IN ABYSSINIAN CATS

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ABSTRACT

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Cell cultures prepared from the spleen and mesenteric lymph nodes of affected kittens were subjected to histochemical and ultrastructural studies. Macrophages in the cultures contained lipid in the cytoplasm and the ultrastructural studies revealed lysosomes containing lamellae similar in appearance to phospholipids.

Résumé

ÉTUDES DE CULTURES DE TISSUS SUR UNE MALADIE LYSOSOMIALE D'EMMAGASINAGE CHEZ LES CHATS ABYSSINIENS

Des cultures de cellules préparées à partir de la rate et des ganglions lymphatiques mésentérique de chatons affectés ont été soumises à des études histochimiques et à microscopie électronique. Les macrophages dans les cultures contenaient du lipide dans le cytoplasme et des études en microscopie électronique révélèrent des lysosomes contenant des lamellae similaires en apparence aux phospholipides.

INTRODUCTION

Lange, Bland van den Berg & Baker (1977) described a suspected lysosomal storage disease in Abyssinian cats, a disease characterized clinically by incoordination, body tremors and sometimes seizures. It was also found to be genetic, with an autosomal recessive mode of inheritance (Bland van den Berg, Baker & Lange, 1977). Histological lesions included vacuolization of neurones and macrophages, and an ultrastructural examination of these cells showed lamellated membranous cytoplasmic bodies (Lange *et al.*, 1977). At the time, the nature of the stored substance in lysosomes could not be determined in formalin-fixed and frozen sections. In an attempt to identify the substance, spleen and lymph node cells of affected kittens were cultured and subjected to histochemical and ultrastructural studies.

MATERIALS AND METHODS

At various times 5 affected kittens were killed by the intravenous administration of an overdose of pentobarbitone sodium. The spleen and mesenteric lymph nodes were removed aseptically within minutes of the kittens being killed. The spleen and lymph nodes were cut into small pieces and the excess blood rinsed off with a calcium- and magnesium-free phosphate-buffered saline solution (PBS) to which penicillin at a concentration of 200 000 units/l and 200 mg streptomycin/l had been added. The tissues were then stirred in the above solution for 30 minutes and the fluid decanted. The spleen and lymph node tissue were trypsinized in a 0.25% trypsin* solution for 1 hour and the cell suspension centrifuged at 200 rpm. The supernatant was removed and the cells were placed in 30 ml disposable tissue culture flasks containing Hanks-Eagle's medium** to which 10% inactivated bovine serum and 1.5 gm/l sodium bicarbonate had been added. The flasks were incubated for 24 hours at 37 °C and the medium was

subsequently decanted and the cultures washed twice in phosphate buffered saline. Fresh medium containing serum was replaced in the flasks which were then returned to the incubator.

Daily, after 4-6 days incubation, individual flasks were removed from the incubator and the following procedures carried out:

(i) The medium from a flask was decanted and the cells fixed to the wall of the flask by adding Bouin's fixative. After 60 minutes the cells in the flasks were stained with the haematoxylin and eosin (HE), oil-red-O (Anon., 1968) and Sudan IV (Lillie, 1944) methods. The top and sides of the flasks were removed and the stained cultures were then examined with the light microscope.

(ii) The other flasks containing 4-6-day-old cultures were prepared for electron microscopy. The medium was decanted and the cultures were washed twice in PBS. The cells were removed with the aid of a trypsin versene solution* and centrifuged for 3 minutes at 2000 rpm. The supernatant was removed, the cells fixed in 2% glutaraldehyde in Millonig's phosphate buffer (pH 7.2-7.4) for 1 hour at 4 °C, washed once in the same buffer and post-fixed for a further hour in 2% osmium tetroxide. This was followed by 2 more washes in buffer, after gentle centrifugation and a resuspension of cellular material. After the final wash, the cells were re-suspended in a small quantity of buffer solution and drawn into non-heparinized micro-haematocrit tubes. The tubes were sealed and centrifuged at high speed to create a plug of compressed cellular material. The plugs were removed from the tubes, cut into small blocks with a sharp blade, dehydrated in ethanol and propylene oxide and embedded in Epon 812 in gelatin capsules for 48 hours at 60 °C. Gold and silver sections were cut with a glass knife on a Reichert OMU 4 Ultracut ultramicrotome, stained in 1% uranyl acetate for 10 minutes and 0.2% lead citrate for 30 seconds and examined in a Philips EM 301 electron microscope.

(iii) Semi-thin sections were cut from the above blocks and stained with toluidine blue for examination with the light microscope.

* Trypsin 1 : 250. Difco Laboratories, Detroit, Michigan, USA

** 50% Hanks balanced salt solution plus 50% Eagle's Minimal Essential Medium

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* 1% Trypsin and 0.4% versene solution

Normal feline lung tissue cultures, 4–6 days old were fixed and stained for light microscopy as described above.

RESULTS

Light microscopy

Cell culture from affected kittens

The growth of the spleen and lymph node cell cultures varied considerably, though on the whole the cultures of the spleen grew much better than those of the lymph nodes. Two distinct growth phases were noticed in these cultures. In the 4-day-old cultures there were a few fibroblast-like cells which were overlaid by many large macrophage-like cells. The macrophage-like cells had large vesicular nuclei with 1 or sometimes 2 distinct nucleoli. The cells were usually rounded, but sometimes amoeboid-like projections were present. In cultures stained with HE the cytoplasm of the macrophage-like cells was filled with numerous round to oval reddish-pink "globules" which varied in size from very small to almost the size of the nucleus (Fig. 3). These cytoplasmic structures stained orange-red to dark-red with the oil-red-O staining method (Fig. 2 & 3), while they stained less intensely with the Sudan IV method. The cytoplasm of the macrophages was filled with many fine granular structures. A few macrophages showed, in addition, a small number of large yellowish-red structures interspersed between the granules (Fig. 4). The second growth phase was observed in the 6-day-old cultures. The fibroblast-like cells predominated, but a few macrophage-like cells, similar to those described in the 4-day-old cultures, were still present. In the older cultures some of these cells showed distinct degenerative changes; the nuclei were small and pyknotic and the plasma membrane of some appeared to have ruptured.

Feline lung cell cultures

After 4 days the flasks had an even growth of fibroblast-like cells which were of the same size and had very large vesicular nuclei with distinct nucleoli. Many of the cells had mitotic figures and some cells were in telophase. The cytoplasm was stellate in shape and only a few small, light-pink granules were seen in the cells stained with HE. These granules were an even red colour with oil-red-O (Fig. 1) and orange with Sudan IV stains.

Semi-thin sections stained with toluidine blue

Most of the cells in the pellets prepared for electron microscopy appeared to be macrophages with a round cytoplasm and a few cytoplasmic projections. The cytoplasm contained structures which varied in morphology with the age of the cultures from which they were prepared. Most of the cells from the 4-day-old cultures had small, uniformly round structures in the cytoplasm, which stained navy blue. A few cells from the 4-day-old cultures and more from the 5-day-old cultures had, in addition, round structures of varying size in the cytoplasm. These stained metachromatically with toluidine blue. Furthermore, many of the macrophages in the 6-day-old cultures contained empty vacuoles of varying sizes in the cytoplasm in addition to the navy blue and metachromatic granules.

Ultrastructure

All the cells examined were round, probably due to the trypsinization process required to release them

from the tissue culture flasks. These cells appeared to be of 2 basic types: some had very large nuclei and a relatively small cytoplasm which, in addition to the usual cytoplasmic organelles, had a few lipid-lysosomes. These lysosomes were surrounded by a single membrane and had a uniform light grey colour. These cells probably correspond to the fibroblast-like cells mentioned earlier. The other cells, to be described below, were similar to the macrophage-like cells found in the tissue cultures and had lipid-containing lysosomes as well as lamellated membranous cytoplasmic bodies which varied in size and shape (Fig. 5). The lamellae were irregularly spaced and in some there were small electron-lucent areas where the lamellae appeared to have drawn apart or disintegrated. Other cells had lysosomes that were dark-grey in colour and were smaller and more abundant than those described above (Fig. 6). In these cells there were also distinct secondary lysosomes which were surrounded by a trilaminar membrane and which contained regularly spaced lamellae, giving an onion-ring appearance (Fig. 7). Cells with these structures were most common in the 4-day-old tissue cultures. In other macrophage-like cells the regularly lamellated structures had fused to form larger irregular bodies with lamellae in different stages of formation and disintegration surrounded by a trilaminar membrane (Fig. 8). In some of these bodies there were areas of a uniform grey colour partially encapsulated by regular lamellae (Fig. 9). Distinct lamellated patterns may be present in the same body. Vacuoles of different sizes containing a few remnants of lamellae in an electron-lucent background were present in other similar membranous cytoplasmic bodies (Fig. 9). Cells with these bodies were more abundant in the 5- and 6-day-old cultures. In addition to the cells described above, there were also cells with the following characteristic appearance: light-grey lysosomes in the cytoplasm and numerous electron-dense, round, lamellated structures of different sizes in which the lamellae were interspersed with a homogeneous electron-lucent, and a light grey granular substance (Fig. 10). This entire structure (lamellae, electron-lucent and light-grey granular substance) was enclosed in a trilaminar membrane (Fig. 11 & 12). In these cells the other cytoplasmic organelles appeared to be affected by degenerative changes. The granular endoplasmic reticulum was dilated and the mitochondria had disappeared from some cells. The nuclei of some of these cells also appeared to have degenerated.

DISCUSSION

Lange *et al.* (1977) described macrophages and neurons with vacuoles in the cytoplasm but the nature of the substance in the vacuoles could not be determined by histochemical methods. In this study, structures present in the cytoplasm of cultured macrophage-like cells stained positive for lipids with oil-red-O and Sudan IV. Lung cell cultures from normal cats were examined simultaneously with the macrophage cultures to determine the amount of lipid present in them. The fibroblast-like cells in these control cultures contained a few lipid droplets, but these were much smaller and stained more evenly than the lipid accumulated in the lysosomes of the macrophage-like cells from affected kittens.

Ultrastructural examination of the cell cultures revealed structures similar to those described previously in the tissues of cats suffering from this disease (Lange *et al.*, 1977).

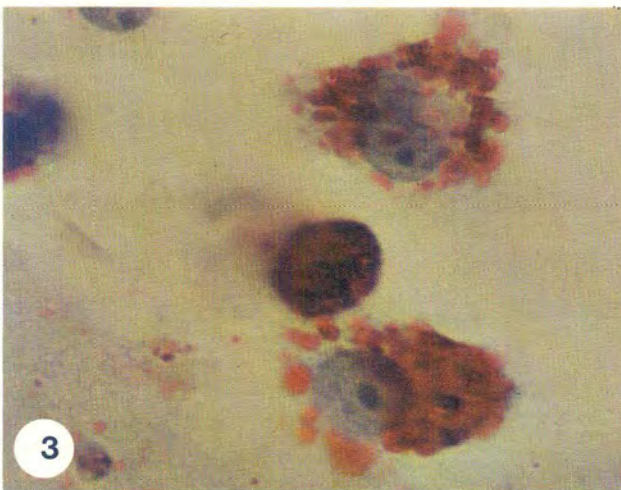
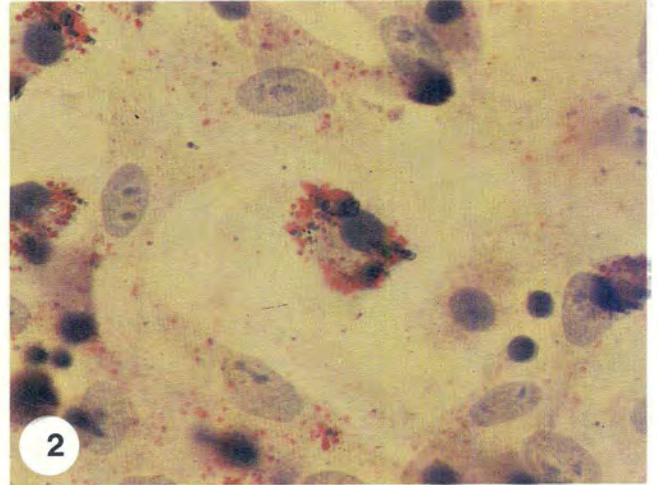
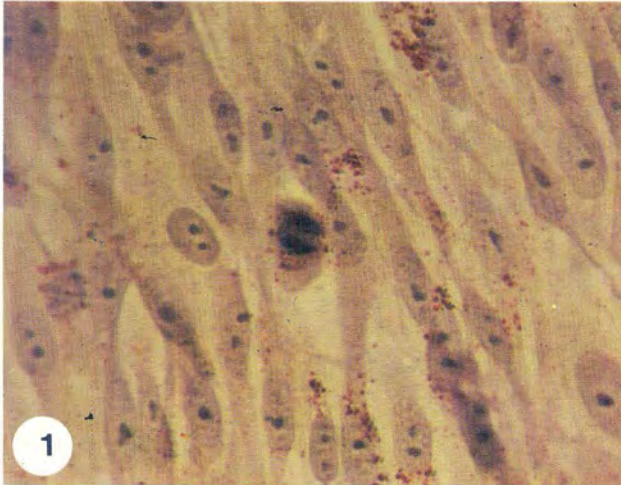
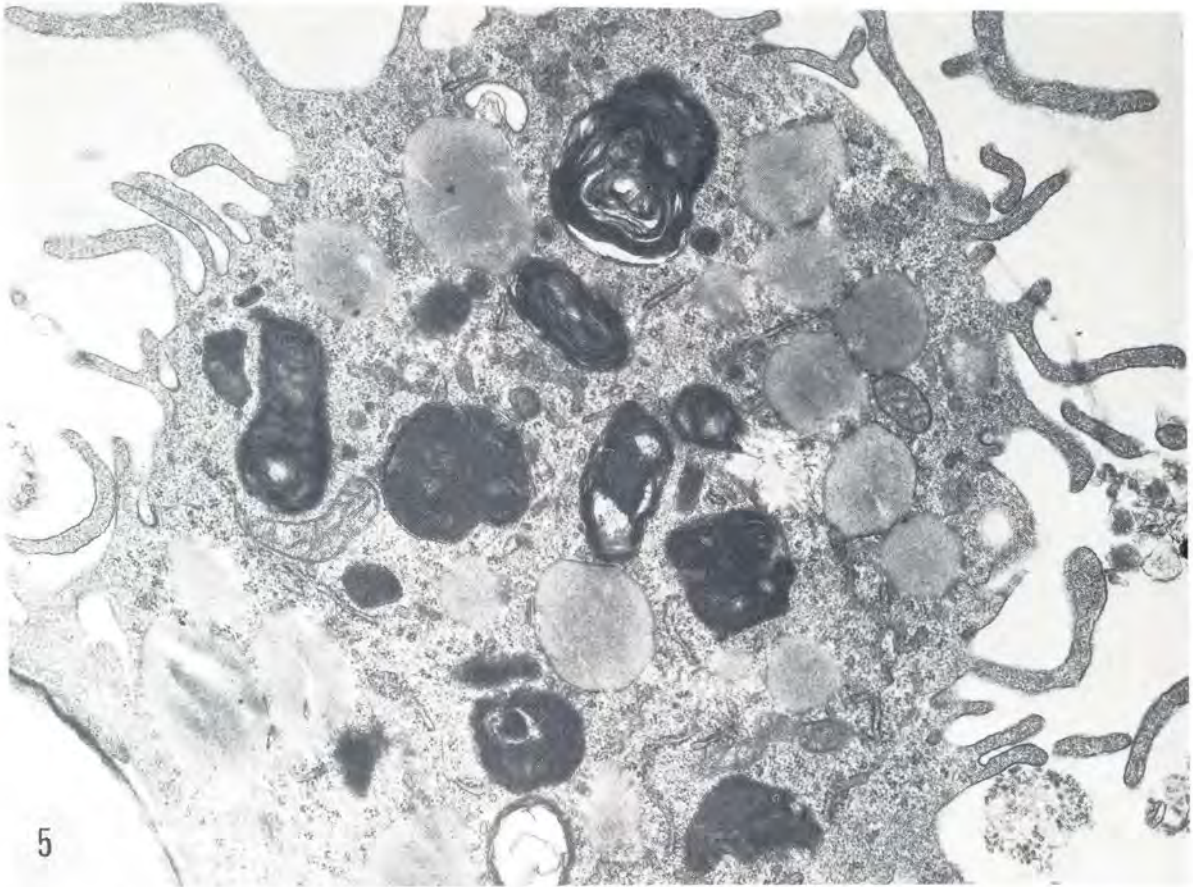


FIG. 1 Normal lung cell culture, 4 days old. Oil-red-0; $\times 800$

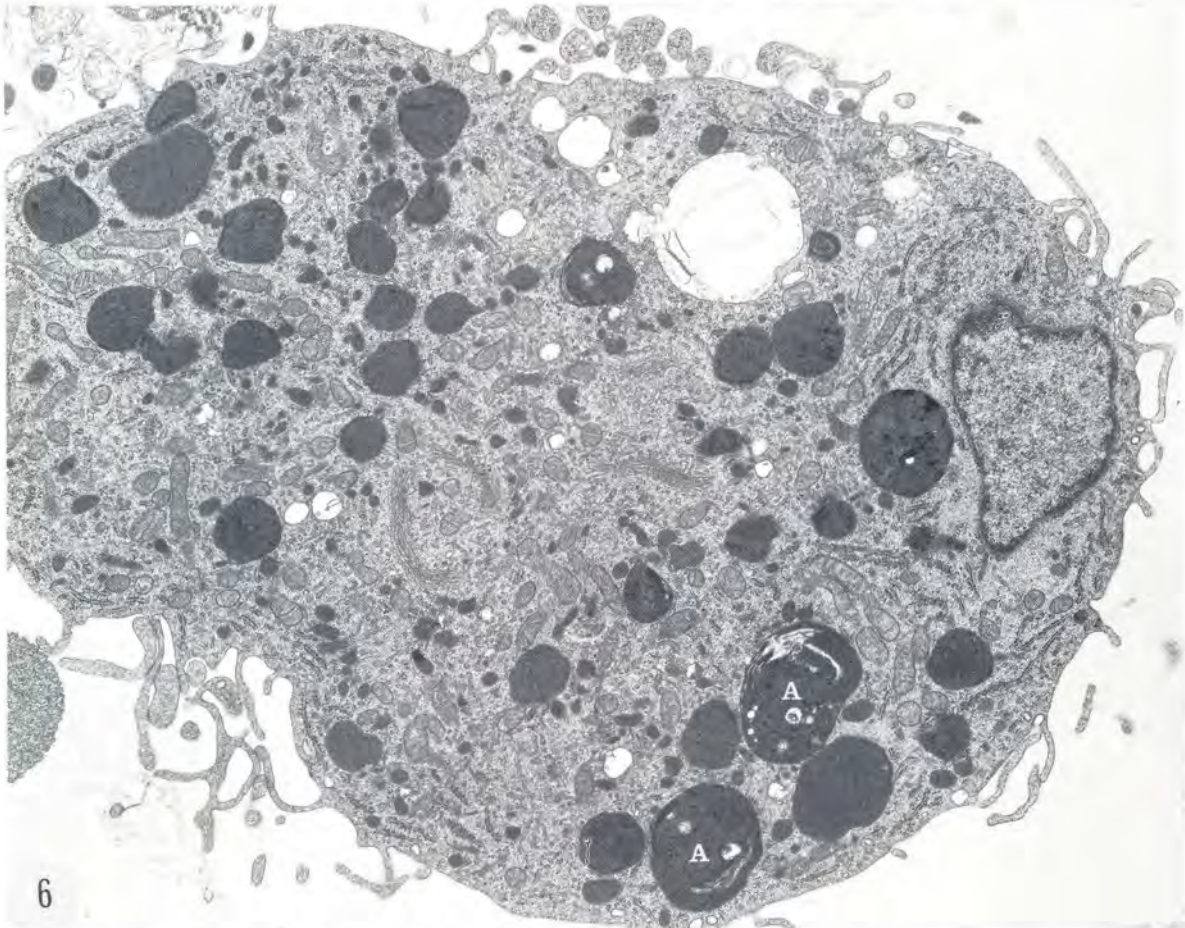
FIG. 2 Four-day-old spleen tissue culture from an affected kitten. Oil-red-0 positive material in the cytoplasm of a macrophage-like cell; Oil-red-0 $\times 800$

FIG. 3 Four-day-old spleen tissue culture from an affected kitten. Oil-red-0 positive structures of varying size in the cytoplasm of macrophage-like cells; Oil-red-0 $\times 2\ 000$

FIG. 4 Six-day-old spleen tissue culture from an affected kitten. One large macrophage-like cell with Sudan IV positive material in the cytoplasm; Sudan IV $\times 2\ 000$



5



6

FIG. 5 A macrophage-like cell from a 4-day-old lymph node tissue culture from an affected kitten. The cytoplasm contains large, light-grey, lipid-containing lysosomes and lamellated membranous cytoplasmic bodies; $\times 17\ 800$
FIG. 6 A macrophage-like cell with many dark-grey lysosomes as well as secondary lysosomes with a lamellated appearance (A); $\times 10\ 600$

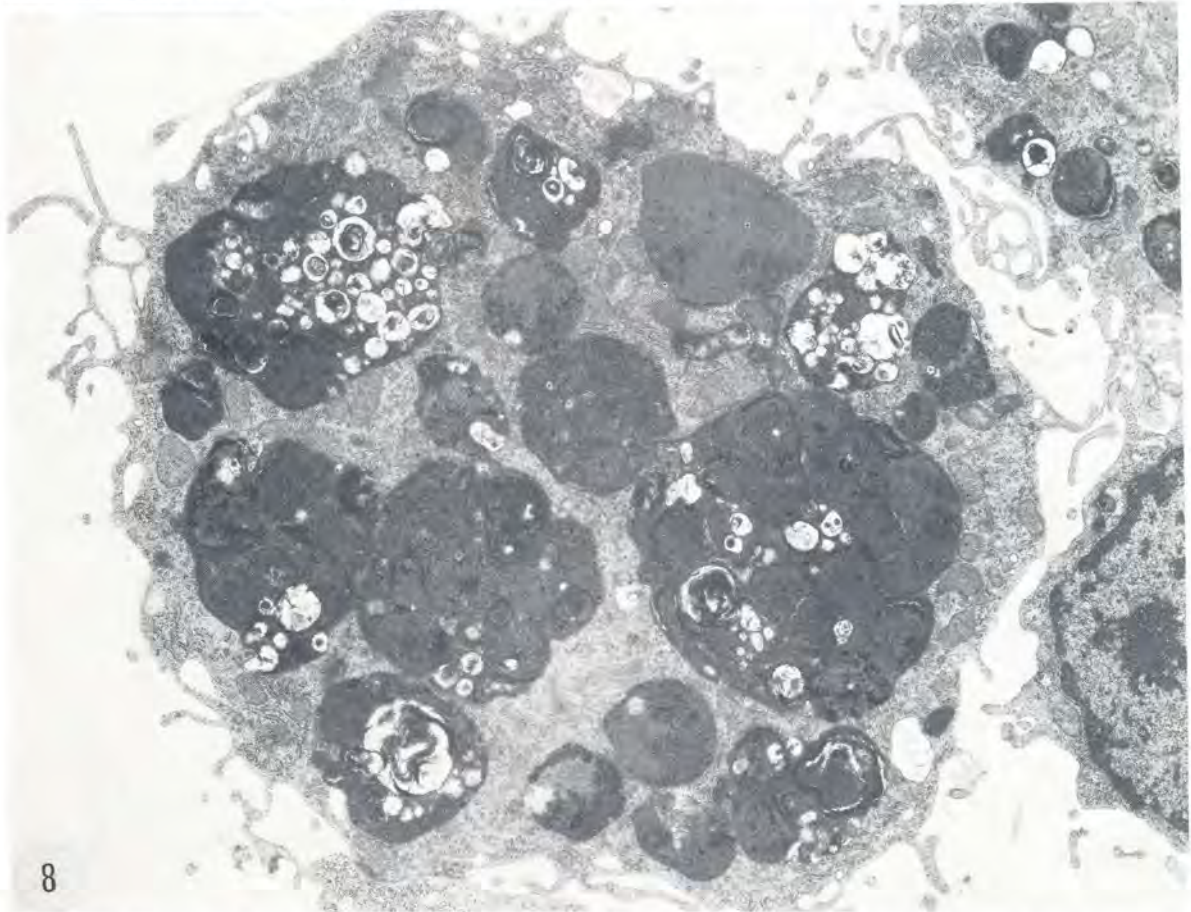
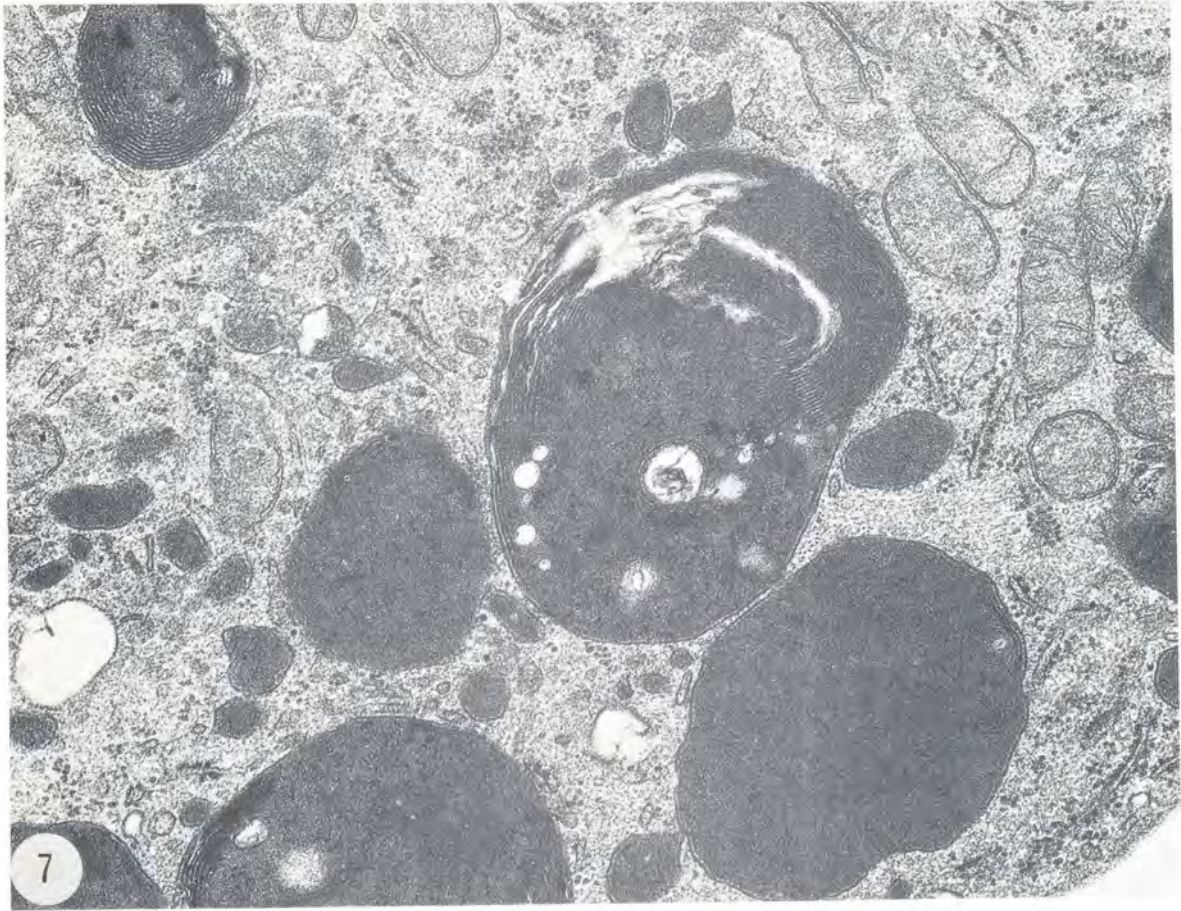
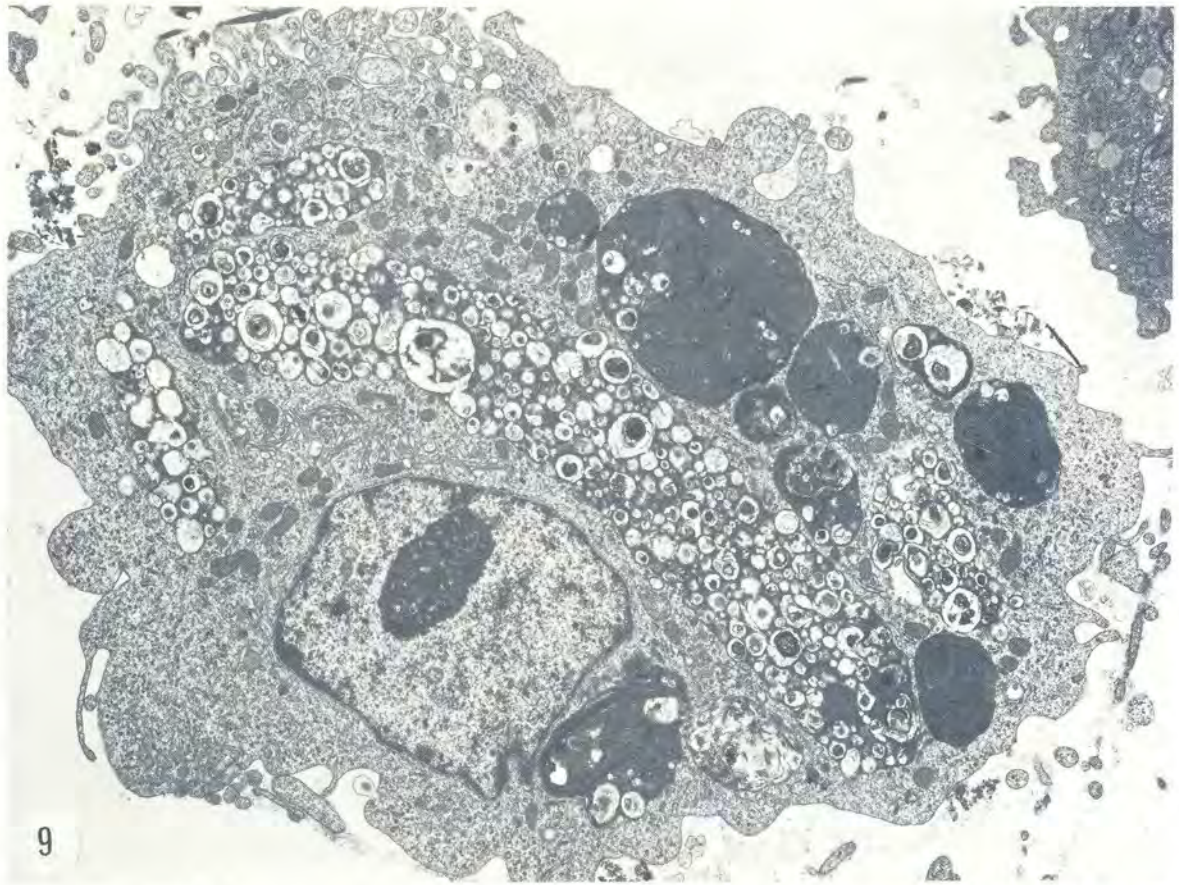
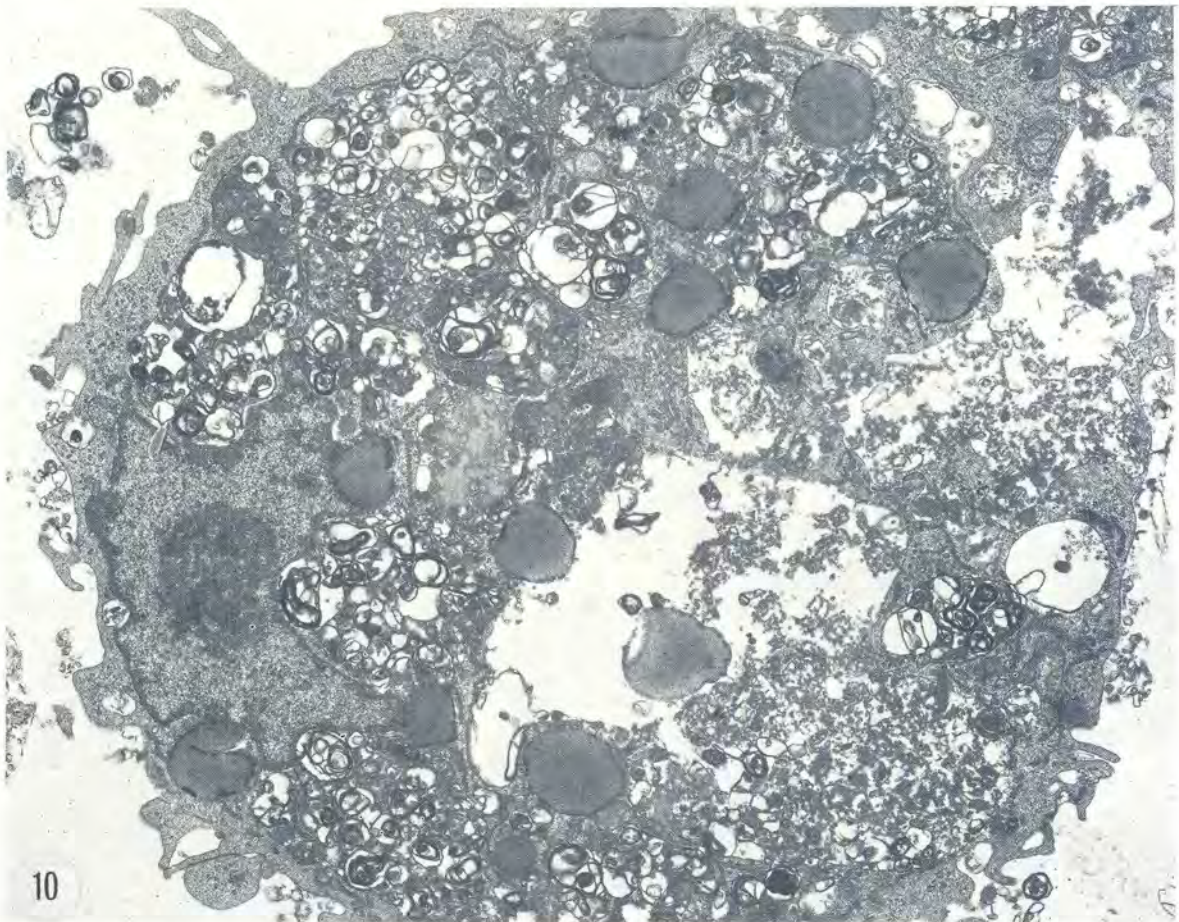


FIG. 7 A membranous cytoplasmic body surrounded by a trilaminar membrane and containing regularly spaced lamellae; $\times 36\ 000$
FIG. 8 A macrophage-like cell illustrating fusion of regularly lamellated structures to form large irregular bodies; $\times 10\ 400$



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FIG. 9 A macrophage-like cell from 5-day-old spleen tissue culture, with large membranous cytoplasmic bodies. Small vacuolated structures surrounded by regular lamellae can also be seen; $\times 8\ 000$

FIG. 10 A cell from a 6-day-old spleen tissue culture containing light-grey lipid lysosomes and numerous electron-dense, round, lamellated structures of different sizes. The lamellae are interspersed with a homogeneous electron-lucent and a light-grey granular substance; $\times 10\ 500$

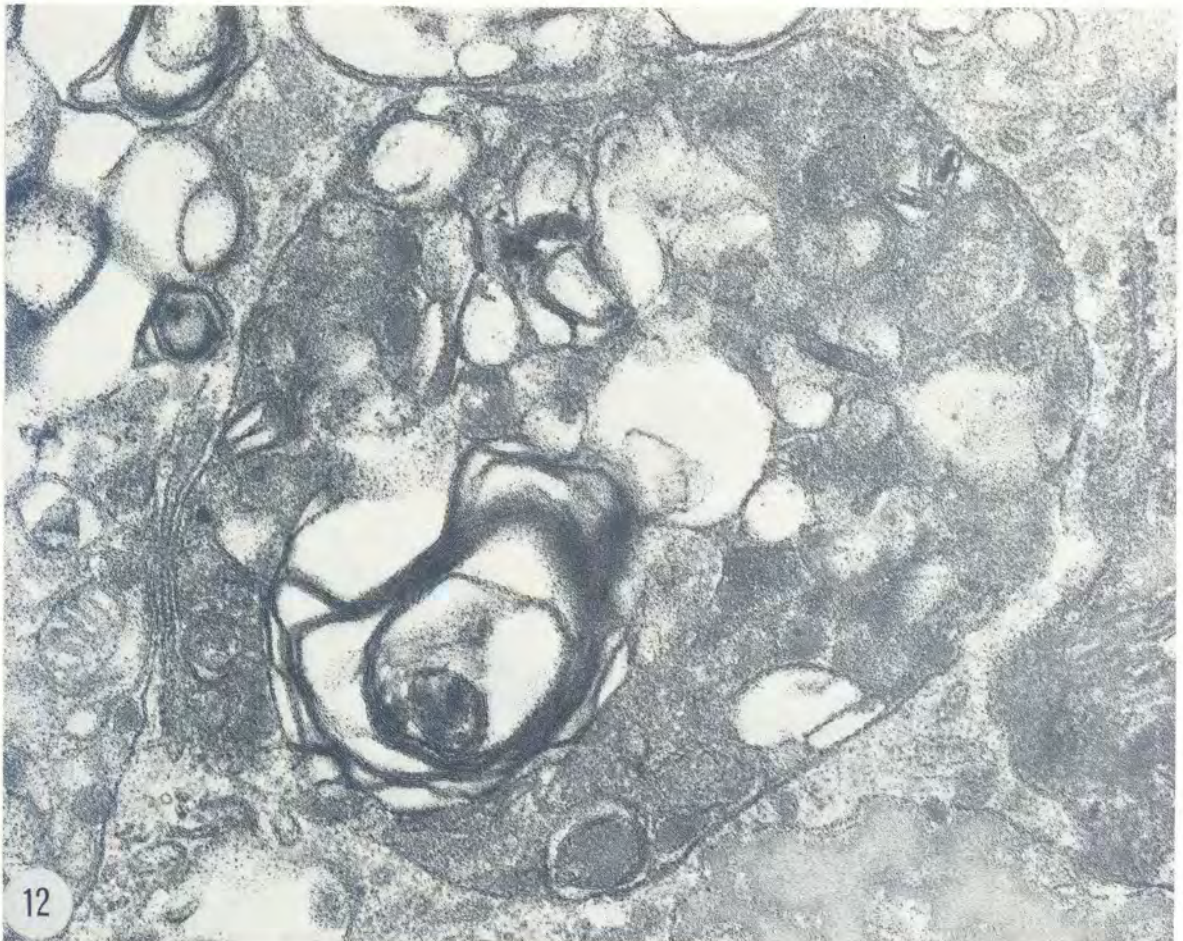
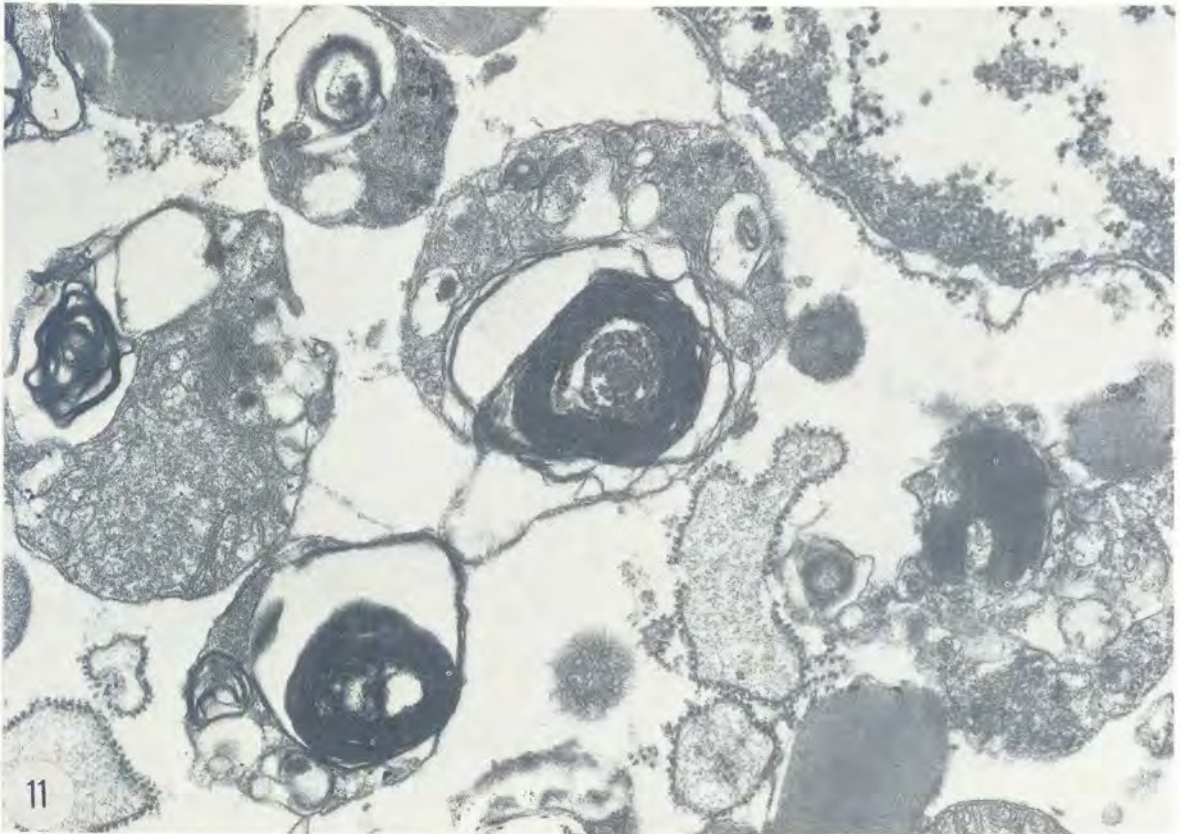


FIG. 11 & 12 Cytoplasmic structures with electron-dense lamellated areas interspersed with electron-lucent and light-grey granular substance. These structures as a whole are surrounded by a trilaminar membrane; $\times 33\ 750$ and $\times 67\ 750$

Light microscopic examination of semi-thin sections stained with toluidine blue revealed the sequential development of the cytoplasmic structures in the macrophages. In the 4-day-old cultures the cell cytoplasm contained small uniformly round navy blue granules, while in the 5-day-old cultures uneven round metachromatic structures were present. In the 6-day-old cultures both these structures were present to a lesser extent, and there were also empty round vacuoles in the cytoplasm. Ultrastructural studies confirmed this pattern of development. Cells in the 4-day-old cultures had lipid-lysosomes in the cytoplasm. In the 5-day-old cultures cells with dark-grey lysosomes were more numerous and more conspicuous than in the former cells. It was also in these cells that the regularly lamellated structures became visible (Fig. 6 & 7). These lysosomes probably correspond to the navy blue granules in the cells of the toluidine blue stained 4- and 5-day-old cultures. As the cells matured, the lamellated membranous bodies fused and started showing signs of disintegration (Fig. 8). At this stage the cells would be comparable to the metachromatic granules mentioned above, since they consist of substances with different electron density. Most of the cells in the 6-day-old cultures had numerous rounded electron-dense lamellated structures of different sizes in the cytoplasm (Fig. 9 & 10). Some of these lamellated bodies appeared dilated and sometimes round electron-lucent areas were seen in these structures. This could be correlated with the vacuoles seen in the cells of 6-day-old cultures stained with toluidine blue. Similar structures were found by Aubert-Tulkens, Van Hoof & Tulkens (1979), when lysosomal phospholipidosis was induced by gentamicin in cultured rat fibroblasts.

The histochemical results and ultrastructural studies of affected cells indicate that the storage disease of Abyssinian cats under investigation is caused by an

enzyme deficiency which results in the accumulation of lipid in lysosomes. When the ultrastructural appearance of the lysosomes is compared with those described by Aubert-Tulkens *et al.* (1979), it would appear that the accumulated lipid could be a phospholipid, but final confirmation of this can only be obtained by biochemical analyses.

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